RESEARCH ARTICLE

Generation and characterization of *Brassica rapa* ssp. *pekinensis* – *B. oleracea* var. *capitata* monosomic and disomic alien addition lines

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Abstract

Five monosomic alien addition lines (MAALs) of *Brassica rapa* ssp. *pekinensis* – *B. oleracea* var. *capitata* were obtained by hybridization and backcrossing between *B. rapa* ssp. *pekinensis* (female parent) and *B. oleracea* var. *capitata*. The alien linkage groups were identified using 42 *B. oleracea* var. *capitata* linkage group-specific markers as *B. oleracea* linkage groups C2, C3, C6, C7 and C8. Based on the chromosomal karyotype of root tip cells, these five MAALs added individual chromosomes from *B. oleracea* var. *capitata*: chr 1 (the longest), chr 2 or 3, chr 5 (small locus of 25S rDNA), chr 7 (satellite-carrying) and chr 9 (the shortest). Five disomic alien addition lines were then generated by selfing their corresponding MAALs.

[Gu A. X., Shen S. X., Wang Y. H., Zhao J. J., Xuan S. X., Chen X. P., Li X. F., Luo S. X. and Zhao Y. J. 2015 Generation and characterization of *Brassica rapa* ssp. *pekinensis* – *B. oleracea* var. *capitata* monosomic and disomic alien addition lines. *J. Genet.* **94**, 435–444]

Introduction

Monosomal alien addition lines (MAALs) contain a single chromosome from one species plus the entire diploid complement of another species as background. In plant breeding programmes, they serve as useful tools for transferring desired genes from a donor to the genome of the recipient crop species. This is accomplished through introgression and/or the development of stable disomic alien addition lines (DAALs) (Khush 2010; Geleta *et al.* 2012). The genus *Brassica* is of worldwide agronomic importance. It comprises three diploid species: *B. rapa* ($2n = 2x = 20$, AA), *B. nigra* ($2n = 2x = 16$, BB) and *B. oleracea* ($2n = 2x = 18$, CC). The *B. rapa* – *B. oleracea* MAALs have one chromosome from *B. oleracea* plus the entire diploid complement of *B. rapa* as background ($2n = 21$, AA + 1C) (Quiros *et al.* 1987; Chen *et al.* 1988, 1992, 1997; McGrath and Quiros 1990; McGrath *et al.* 1990; Hu and Quiros 1991; Cheng *et al.* 1994, 1995; Heneen and Jørgensen 2001; Geleta *et al.* 2012). By analysing these *B. rapa* – *B. oleracea* MAALs, researchers have been able to map the genes on *B. oleracea* chromosomes for seed colour (Heneen and Brismar 2001; Heneen *et al.* 2012), white flowers (Cheng *et al*. 1995, Chen *et al*. 1997), erucic acid content, and the *LAP-lCc* locus of the leucine aminopeptidase isozyme (Chen *et al.* 1992; Cheng *et al.* 1995) One set of these MAALs (Chen *et al.* 1992, 1997; Geleta *et al.* 2012) was developed using the parental pair of *B. rapa* var. *trilocularis* (Yellow Sarson, K-151) and *B. oleracea* var. *alboglabra* (no. 4003). Other MAALs were obtained from *B. rapa* (Swedish breeding line sv85-3801) and no. 4003 (Chen *et al.* 1992; Cheng *et al.* 1995). The creation of these MAALs involved using diploid *B. oleracea* as the female crossed with diploid *B. rapa*. The allotetraploid *B. napus* (AACC) was obtained through chromosome doubling. Later, sesquidiploids (genome AAC) were produced by backcrossing *B. napus* and *B. rapa*, then selfing or backcrossing to *B. rapa*. Some MAALs were generated from crossing between the resynthesized *B. napus* (Hakuran B454), hybridizing *B. oleracea* as the female with *B. rapa* (Nishi 1980) and recurrent parents 'Torch' (*B. rapa* ssp. *olifera*; accession B200) and 'Kwan Hoo Choi' (*B. rapa* ssp. *parachinensis*; accession B233) (McGrath *et al.* 1990). A few MAALs were created from crosses between natural *B. napus* and diploid *B. rapa* ('Torch', 'Kwan Hoo Choi'; rapid cycling CrGC-01, CrGC-13), then backcrossing to the diploid (Quiros *et al.* 1987). Alien addition lines obtained have cytoplasm from *B. oleracea* or natural *B. napus*; their background genomes are those of *B. rapa* that host

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Keywords. monosomic alien addition line; disomic alien addition line; linkage group; chromosome; *Brassica rapa* ssp. *pekinensis*; *B. oleracea* var. *capitata*.

individual *B. oleracea* chromosomes. All of the *B. rapa* var. *trilocularis* (K-151) – *B. oleracea* var. *alboglabra* (no. 4003) MAALs are generally characterized as weaker in vigour, stature and fertility when compared with the *B. rapa* parent (Heneen *et al.* 2012). This possibly results from nuclear–cytoplasmic interactions except for genes from the alien *B. oleracea* var. *alboglabra*.

Brassica rapa ssp. *pekinensis* (Chinese cabbage) has an important role in human diets, particularly in China, where the argument is made that 'one-hundred vegetables are inferior to Chinese cabbage'. It is also an essential ingredient of the very popular Kimchi in Korea. Whereas *B. rapa* ssp. *pekinensis* is seed vernalization responsive, *B. oleracea* var. *capitata* is plant vernalization responsive. The latter species show stronger resistance to downy mildew and TuMV than the former, and its levels of glucosinolates are also 3–20 times higher (Cartea *et al.* 2008; Chen *et al.* 2008). The byproducts of glucosinolates provide plant defenses against pests, food flavourings, and benefits to human health, such as being an anticarcinogen (Mithen *et al.* 2003; Brew *et al.* 2009). Because of these properties, we designed the current study to focus on the production of MAALs that utilize the cytoplasm and entire diploid complement from *B. rapa* ssp. *pekinensis* and add one chromosome from *B. oleracea* var. *capitata*. Our objective was to develop chromosome segment introgression lines (CSILs) for *B. rapa* ssp. *pekinensis* – *B. oleracea* var. *capitata*, in which nuclear–cytoplasmic incompatibilities were eliminated so that we could analyse how alien genes or even a chromosome segment influences the manifestation of desired characteristics.

Materials and methods

Plant materials

The plant materials include inbred line *Brassica rapa* ssp. *pekinensis* (85-1; AA, $2n = 20$) and the inbred line *B. oleracea* var. *capitata* (11-1; CC, $2n = 18$). These lines were produced by selfing and were selected over six generations. Autotetraploidy for 85-1 (AAAA, $2n = 40$) was achieved by treating the stem apexes with a 0.1% colchicine solution (Zhang *et al.* 1999). Sesquidiploid hybrids (AAC, $2n = 20 + 9 = 29$) were produced by crossing the autotetraploid *B. rapa* ssp. *pekinensis* and the diploid *B. oleracea* var. *capitata* by the ovary and ovule culture method (Gu *et al.* 2006). In our study, these AAC hybrids served as the nonrecurrent parent while 85-1 was the recurrent parent. The scheme for AAC hybrid production, and selection of MAALs and DAALs are shown in figure 1.

Chromosome preparations

For MAAL selection, seeds from BC_2F_1 were sown on a 1/2 MS medium. Root tips from the resultant seedlings were excised and immersed in 2 mM 8-hydroxyquinoline for 2 h, then fixed for 24 h using Carnoy's solution (3 : 1, 95% ethanol : glacial acetic acid). Chromosomes from the root tip cells were prepared by the common squash method. The samples were observed under an Olympus BH-2 microscope (Olympus, Tokyo, Japan) with a 100× Plan Apo oil-immersion lens. The corresponding stem apexes from these seedlings were transferred temporarily to a standard MS medium.

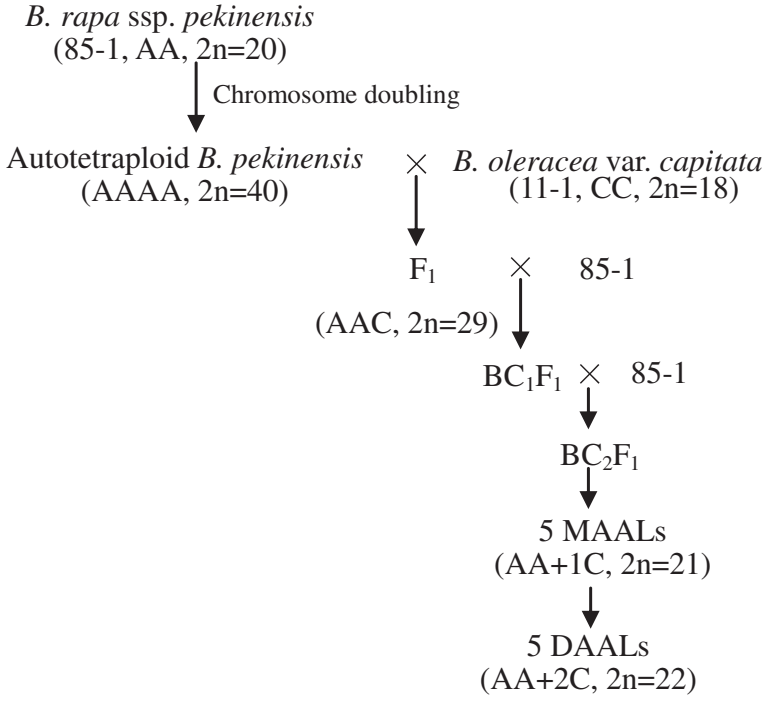

Figure 1. Breeding scheme for development of MAALs and DAALs.

Designation of linkage group-specific markers

Our genetic linkage groups (LGs) were in accord with the internationally agreed numerical designation system for chromosomes of the *Brassica* genome (http://www.brassica.info/resource/maps/lg-assignments.php). Simple sequence repeats (SSRs) were demonstrated to be specific to only one of the nine C-linkage groups in *B. oleracea* or *B. napus* (N11–N19). (http://www.brassica.info/resource/markers/ssr-exchange.php#map_locations; Piquemal *et al.* 2005) were screened for their specificity to *B. oleracea* var. *capitata* compared with *B. rapa* ssp. *pekinensis*. We used DNA samples from eight individual plants of *B. oleracea* var. *capitata* and seven of *B. rapa* ssp. *pekinensis*. Specificity was defined as the presence of PCR products in *B. oleracea* var. *capitata* with no product being detected in *B. rapa* ssp. *pekinensis* or *B. oleracea* var. *capitata* and having polymorphisms of lengths comparable to those of *B. rapa* ssp. *pekinensis*. We obtained 37 LG-specific markers of *B. oleracea* var. *capitata* (Gu *et al.* 2009). When only a few specific markers were available, we applied expressed sequence tag (EST)-SSR markers to the LG (Chen *et al.* 2013; Qin 2013). Each LG of *B. oleracea* had four to seven specific markers.

Identification with linkage group-specific markers

Linkage group-specific markers were used to identify plants with $2n = 21$, and the alien addition LGs were determined from *B. oleracea* var. *capitata*. Genomic DNA was extracted from the leaves of parents as well as plants with $2n = 21$ using CTAB method. DNA amplifications were performed in 20 μL volumes that contained 1 U of *Taq* polymerase, 0.2 μM of primers, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, and 50–150 ng of genomic DNA as the template. The PCR conditions included an initial 3 min at 95°C; then 35 cycles of 1 min for DNA denaturation at 94°C, 30 s at 55–60°C and 45 s for extension at 72°C, with a final extension of 5 min at 72°C. The amplification products were detected on 6% polyacrylamide gels and DNA bands were visualized by silver staining.

Karyotype analysis

To conduct the karyotype analysis of $2n + 1$ plants with the added LG of *B. oleracea* var. *capitata* and the parents, we utilized mitotic metaphase chromosomes from the root tip cells. Samples were observed under an Olympus BH-2 microscope with a 100×Plan Apo oil-immersion lens. Images were captured using the Olympus Microsuite 5 software package Olympus, Tokyo, Japan). Chromosome arrangements for the karyotypes of MAALs and parents followed conventional systems that included parameters for chromosome size, arm ratio and the presence or absence of satellites (Li and Zhang 1996).

Fluorescence *in situ* hybridization (FISH) was performed using 25S rDNA sequences as probes on two MAALs and the parents. Somatic chromosomes were prepared as described by Song and Gustafson (1995). A 2.3-kb ClaI subclone of the 25S rDNA coding region of *Arabidopsis thaliana* was labelled by random priming with digoxigenin-11-dUTP (Roche, Penzberg, Germany), according to the manufacturer's instructions. *In situ* hybridization was conducted as described by Xuan *et al.* (2007).

Quantity and viability of pollen

The number of pollen grains per mature anther was determined by first making a pollen suspension liquid (one anther in 1 mL of 10% sucrose solution), then counting the isolated grains with a haemocytometer ($0.0025\ mm^2 \times 0.1$ mm). This procedure was repeated 10 times for each specimen. Pollen viability was defined according to the percentage of pollen grains that were stained with 0.5% triphenyl tetrazolium chloride. Each specimen had more than 500 grains. Normal grains were densely stained (red), while aborted grains were lightly stained or completely colourless.

Production and identification of DAALs

After the MAALs were selfed, the number of somatic chromosomes from the progenies were recorded. The DAALs were screened for chromosome counts, identification of LG-specific markers and karyotype.

Results

Identification with linkage group-specific markers

Our examination of the chromosomes from root tip cells revealed that most plants were $2n = 23$, 24, 25 or 26 in BC_1F_1. We then screened those with $2n = 21$ in BC_2F_1, and verified that 10 of the 135 total seedlings with dispersed and clear chromosomes were $2n = 21$. Those seedlings with $2n = 21$ were analysed by 42 LG-specific markers with nine assigned LGs of *B. oleracea* (table 1). One seedling contained amplified *B. oleracea* var. *capitata*-specific loci and/or alleles in five markers (Ol13G05, sORA43, Na12C03, Na12H09 and BoE020) from LGC2. Also it amplified only *B. rapa* ssp. *pekinensis* loci and/or alleles in the 37 specific markers from the other LGs. This demonstrated that the added chromosome was from LGC2, namely AA+LGC2. Our identification with LG-specific markers showed that two seedlings had an additional LGC3 (AA+LGC3) and one had LGC7 (AA+LGC7). One seedling had amplified *B. oleracea* var. *capitata*-specific loci and/or alleles in four markers from LGC6 and Ol13G05 from LGC2. It also had amplified *B. rapa* ssp. *pekinensis* loci and/or alleles in 37 specific markers from the other LGs, thereby indicating that the added chromosome was mainly from LGC6 (AA+LGC6). One seedling had amplified *B. oleracea* var. *capitata*-specific loci and/or alleles in three out of four markers from LGC8, but only amplified *B. rapa* ssp. *pekinensis* loci and/or alleles in 38 specific markers from the other LGs plus BoE116 from LGC8. This showed that the added chromosome was from LGC8 (AA+LGC8).

Table 1. Amplification of linkage group-specific markers in MAALs and DAALs.

Marker	LG	*B. pekinensis*	*B. capitata*	AA+L GC2	AA+2L GC2	AA+L GC3	AA+2L GC3	AA+L GC6	AA+2L GC6	AA+L GC7	AA+2L GC7	AA+L GC8	AA+2L GC8
Na10H03	C1	1	2	1	1	1	1	1	1	1	1	1	1
Ni4B10	C1	0	2	0	0	0	0	0	0	0	0	0	0
Na12C08	C1	1	2	1	1	1	1	1	1	1	1	1	1
Ol10A11	C1	1	2	1	1	1	1	1	1	1	1	1	1
Ol13G05	C2	0	2	2	2	0	0	2	2	0	0	0	0
sORA43	C2	1	2	2	2	1	1	1	1	1	1	1	1
Na12C03	C2	0	2	2	2	0	0	0	0	0	0	0	0
Na12H09	C2	1	2	2	2	1	1	1	1	1	1	1	1
BoE020	C2	1	2	2	2	1	1	1	1	1	1	1	1
Na12F12	C3	1	2	1	1	2	2	1	1	1	1	1	1
Ol13H09	C3	1	2	1	1	2	2	1	1	1	1	1	1
Ol10B04	C3	1	2	1	1	2	2	1	1	1	1	1	1
Ol11G11	C3	1	2	1	1	2	2	1	1	1	1	1	1
BN12A	C3	1	2	1	1	2	2	1	1	1	1	1	1
BoE028	C3	1	2	1	1	2	2	1	1	1	1	1	1
BoE076	C3	1	2	1	1	2	2	1	1	1	1	1	1
Na12E05	C4	1	2	1	1	1	1	1	1	1	1	1	1
Ol10F12	C4	0	2	0	0	0	0	0	0	0	0	0	0
Ol11H08	C4	1	2	1	1	1	1	1	1	1	1	1	1
Ol12F07	C4	1	2	1	1	1	1	1	1	1	1	1	1
Na10F06	C4	1	2	1	1	1	1	1	1	1	1	1	1
Na12E06	C5	1	2	1	1	1	1	1	1	1	1	1	1
Ni4C11	C5	1	2	1	1	1	1	1	1	1	1	1	1
Ol12F02	C5	1	2	1	1	1	1	1	1	1	1	1	1
Na10D11	C5	0	2	0	0	0	0	0	0	0	0	0	0
Ra2A04	C5	1	2	1	1	1	1	1	1	1	1	1	1
Ra2A05	C6	1	2	1	1	1	1	2	2	1	1	1	1
Ap1a5pr	C6	1	2	1	1	1	1	2	2	1	1	1	1
Na14F11	C6	1	2	1	1	1	1	2	2	1	1	1	1
BoE380	C6	1	2	1	1	1	1	2	2	1	1	1	1
Ol10B01	C7	1	2	1	1	1	1	1	1	2	2	1	1
BN72a	C7	1	2	1	1	1	1	1	1	2	2	1	1
Na12F03	C7	0	2	0	0	0	0	0	0	2	2	0	0
Ol10H04	C7	0	2	0	0	0	0	0	0	2	2	0	0
Ol12D05	C8	1	2	1	1	1	1	1	1	1	1	2	2
Ni4D09	C8	1	2	1	1	1	1	1	1	1	1	2	2
Ol12G04	C8	1	2	1	1	1	1	1	1	1	1	2	2
BoE116	C8	1	2	1	1	1	1	1	1	1	1	1	1
Ol12D09	C9	1	2	1	1	1	1	1	1	1	1	1	1
Ol10D08	C9	1	2	1	1	1	1	1	1	1	1	1	1
Ra1F03	C9	1	2	1	1	1	1	1	1	1	1	1	1
Ol12A04	C9	1	2	1	1	1	1	1	1	1	1	1	1

0, unamplified product; 1, amplified products from *B. rapa* ssp. *pekinensis*; 2, amplified products from *B. oleracea* var. *capitata*.

Identification of the alien B. oleracea var. capitata chromosome in MAALs

To examine any correlation between LGs and chromosomes in *B. oleracea* var. *capitata*, we analysed the karyotype of the five vegetative lines with the additional alien LG from *B. oleracea* var. *capitata* and its parents.

The karyotypes of *B. rapa* ssp. *pekinensis* and *B. oleracea* var. *capitata* were constructed from the FISH results shown in figure 2, a&b, respectively. The chromosomes were numbered according to length, with the size of the satellite being deducted for the satellite-carrying chromosome. In *B. rapa* ssp. *pekinensis*, four pairs of homologous chromosomes had distinct 25S rDNA loci. Among them were two pairs of homologous chr 1 and 3 with 25S rDNA loci in the middle of their long arms; one pair of homologous chr 2 with large 25S rDNA loci (the signal usually encompassing the short arm and satellite); one pair of chr 4 with 25S rDNA loci near the centromere region; and homologous chr 5, which had the least pronounced signals near the centromere region. Based on their arm ratios, we classified the chromosomes as being a metacentric or submetacentric type. Five chromosome pairs (1, 3, 5, 6 and 8) were metacentric while the submetacentric group comprised five other chromosome pairs (2, 4, 7, 9 and 10). In *B. oleracea* var. *capitata*, two pairs of chromosomes had 25S rDNA loci while one pair of the satellite-carrying chr 7 had a 25S locus at the terminal ends of the short arms and satellite. Another pair of chr 5 also had loci at the ends of the short arm, but the signal was generally weaker for chr 7. Four chromosome pairs (2, 3, 6 and 8) were metacentric while five (1, 4, 5, 7 and 9) were submetacentric.

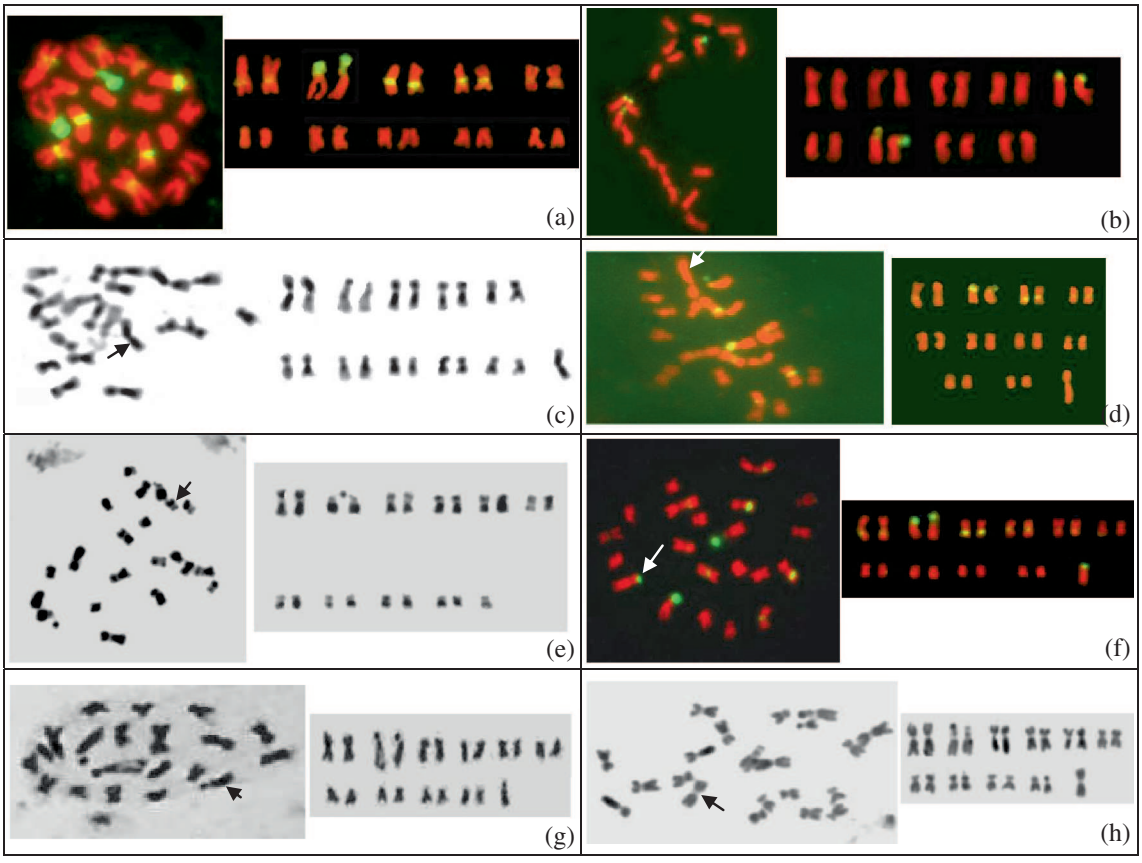

Figure 2. Mitotic chromosomes and karyotypes at metaphase for *B. rapa* ssp. *pekinensis – B. oleracea* var. *capitata* MAALs and parents: (a) FISH conducted using 25S rDNA probe of *B. rapa* ssp. *pekinensis*, (b) FISH conducted using 25S rDNA probe of *B. oleracea* var. *capitata*, (c) AA+LGC2, (d) FISH conducted with 25S rDNA probe of AA+LGC3, (e) AA+LGC6, (f) FISH conducted with 25S rDNA probe of AA+LGC7, (g) AA+LGC8, and (h) AA+LGC4.

The alien chromosome from *B. oleracea* var. *capitata* in the AA+LGC2 genome was morphologically distinct from the *B. rapa* ssp. *pekinensis* chromosomes, i.e. it was metacentric and shorter than chr 1 but longer than chr 3 from *B. rapa* ssp. *pekinensis* (figure 2c). At metaphase, the chromosome from *B. oleracea* var. *capitata* in one cell was longer than the correspondingly numbered chromosome from *B. rapa* ssp. *pekinensis* (Cheng *et al.* 1995; Qie 2007). Therefore, we preliminarily designated this alien chromosome as chr 2 or 3 of *B. oleracea* var. *capitata*. The alien submetacentric chromosome in the AA+LGC3 genome was longer than chr 1 of *B. rapa* ssp. *pekinensis*, and it closely matched chr 1 in size and centromere position (figure 2d) of *B. oleracea* var. *capitata*. Therefore, we concluded that the AA+LGC3 genome harboured chr 1 of *B. oleracea* var. *capitata*. The alien chromosome in the AA+LGC6 genome was submetacentric. It was not chr 8 of *B. oleracea* var. *capitata* because chr 8 was metacentric. Instead, it closely matched chr 9 in size and centromere position (figure 2e). The alien chromosome in the AA+LGC7 genome carried the 25S rDNA locus at the terminal ends of its short arms, evidently matching chr 5 (figure 2f). In AA+LGC8, the alien chromosome was the most easily identifiable since its satellite was smaller than the satellite-carrying chromosome in the background *B. rapa* ssp. *pekinensis* (figure 2g) (Chen *et al.* 1997). Therefore, this alien *B. oleracea* var. *capitata* chromosome in the MAAL was obviously satellite chr 7. Based on the results of analysis for linkage group-specific markers and the MAAL karyotype, we were able to establish the following matches between LGs and chromosomes: LGC2 with chr 2 or 3, LGC3 with chr 1, LGC6 with chr 9, LGC7 with chr 5 and LGC8 with chr 7.

Characterization of MAALs

Five MAALs and their parents were transferred to a 1/2 MS medium for root initiation. Those that rooted (at least five plants per line) were moved to the greenhouse in February. The AA+LG8 plants had the greatest vigour whereas AA+LG6 had the weakest. Variations in leaf and floral organ traits were noticed among the MAALs (figure 3; table 2). For example, leaf type and colour as well as the presence of a waxy leaf surface were similar between AA+LG8 and its parent *B. oleracea* var. *capitata*. Plants of AA+LG6 did not require vernalization to induce flowering and their buds and petals were smaller than those from other MAALs and parents. Two different kinds of angles were found between petal and column (figure 3i). The included angles of AA+LG6

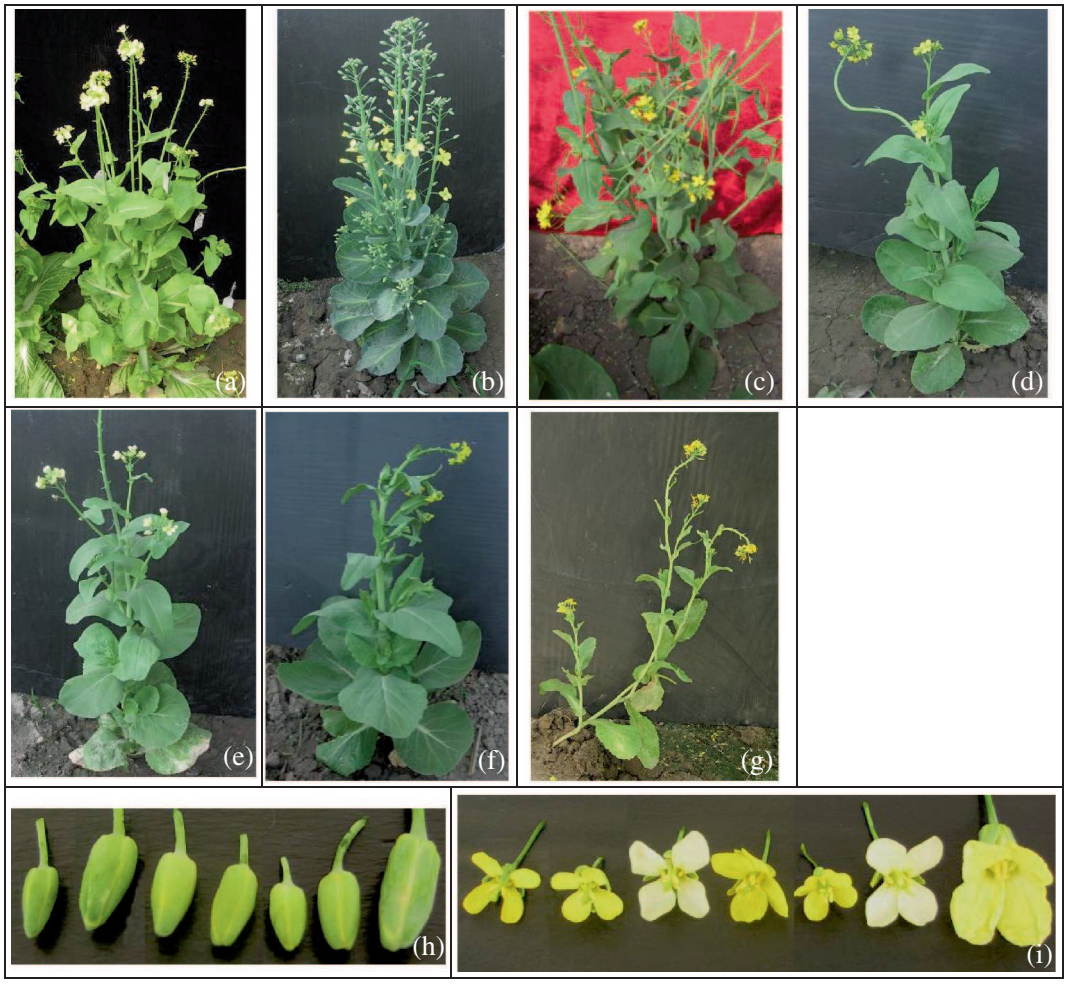

Figure 3. Phenotypes of *Brassica rapa* ssp. *pekinensis* – *B. oleracea* var. *capitata* MAALs and parents (a–g), whole plants of *B. rapa* ssp. *pekinensis* (a), *B. oleracea* var. *capitata* (b), AA+LGC3 (c), AA+LGC2 (d), AA+LGC7 (e), AA+LGC8 (f), and AA+LGC6 (g); (h) buds (l to r): AA+LGC3, AA+LGC2, AA+LGC7, AA+LGC8, AA+LGC6, *B. rapa* ssp. *pekinensis*, and *B. oleracea* var. *capitata*; and (i) flowers (l to r): AA+LGC3, AA+LGC2, AA+LGC7, AA+LGC8, AA+LGC6, *B. rapa* ssp. *pekinensis* and *B. oleracea* var. *capitata*.

and AA+LG8 were smaller and their petals pointed upward, similar to the parent *B. oleracea* var. *capitata*. By contrast, the included angles of AA+LG2, AA+LG3, AA+LG7 and the parent *B. rapa* ssp. *pekinensis* measured almost 90°, and their petals were open and flat. Among the MAALs, the rate at which selfed siliques were set was highest for AA+LG8, which was similar to the parents; while the rate of seed set for selfed AA+LG6 plants was lower than the parents and was lowest for above-mentioned MAALs, with values 18.9 and 14.4%, respectively (table 3).

Developing DAALs

MAALs were selfed so that we could screen their corresponding DAALs. In 30–43 plants obtained from the progenies of each MAAL, chromosome numbers were $2n = 20$ 21 or 22. The percentages of $2n = 22$ in AA+LGC2, AA+LGC3, AA+LGC6, AA+LGC7 and AA+LGC8 were 6.06, 7.89, 5.26, 10.81 and 4.65%, respectively. Their corresponding DAALs were identified by LG-specific markers (table 1) and karyotype analysis (figure 4). They were named AA+2LGC2, AA+2LGC3, AA+2LGC6, AA+2LGC7 and AA+2LGC8.

Several characteristics of DAALs were similar to those of their corresponding MAALs. However, some of their differences included flower traits, with AA+2LGC8 being lighter in colour than AA+LGC8; and petals overlapping for AA+LGC6 but not for AA+2LGC6 (table 2). The DAALs had fewer pollen grains per anther and lower pollen viability when compared with their corresponding MAALs, and values for those traits were lower for the MAALs than for the *B. pekinensis* and *B. capitata* parents (table 3). Except for AA+2LGC6, the rate at which selfed siliques were set was slower for DAALs than for the corresponding MAALs.

Table 2. Main characteristics of MAALs and parents.

Material	Main characteristics
B. pekinensis	Stronger vigour; light green leaf, no wax; white flower, petals open and flat, petal length/width = 1.90; mid-range flowering time
B. capitata	Strong vigour; dark green leaf with thick wax; yellow flower, petals facing upward and overlapping, petal length/width = 1.69; late flowering
AA+LG2, AA+2LG2	Strong vigour; small green leaf, no wax, similar in type to parent *B. pekinensis*; yellow flower, petals open and flat, with no overlap, petal length/width ~1.70; mid-range flowering
AA+LG3, AA+2LG3	Strong vigour; green leaf, no wax; yellow flower, petals open and flat, with no overlap, petal length/width ~2.30; mid-range flowering
AA+LG6, AA+2LG6	Weak vigour; light green leaf, no wax; yellow flower, petals facing upward and overlapping (AA+LG6), petals facing upward but not overlapping (AA+2LG6), petal length/width = 1.75 (AA+LG6) to 1.88 (AA+2LG6); early flowering
AA+LG7, AA+2LG7	Stronger vigour; green leaf, similar in type to parent *B. pekinensis*; white flower, petals open and flat, with no overlap, petal length/width ~1.69; mid-range flowering
AA+LG8, AA+2LG8	Strongest vigour; dark green leaf with wax, similar in type to parent *B. capitata*; yellow flower (AA+LG8), white flower (AA+2LG8); petals facing upward and overlapping, petal length/width = 1.70 (AA+2LG8) to 2.28 (AA+LG8); mid-range flowering

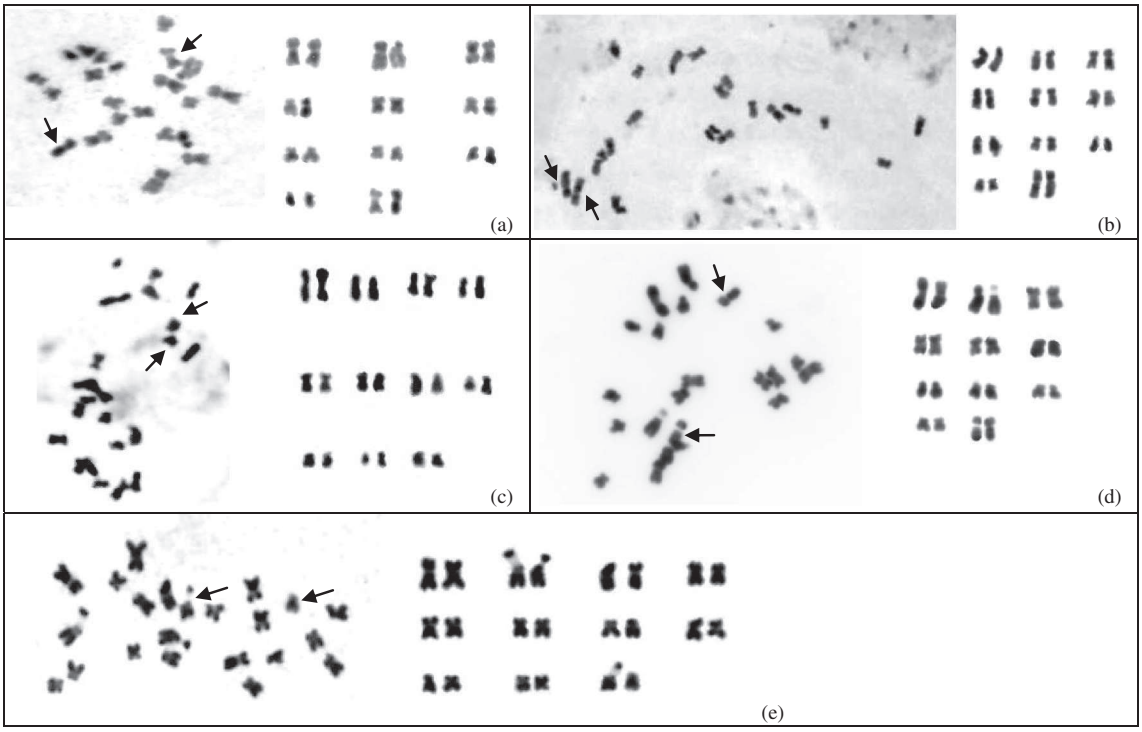

Figure 4. Mitotic chromosomes and karyotypes at metaphase for *B. rapa* ssp. *pekinensis – B. oleracea* var. *capitata* DAAL. (a) AA+2LGC2, (b) AA+2LGC3, (c) AA+2LGC6, (d) AA+2LGC7 and (e) AA+2LGC8.

For all selfed DAALs, the rate of seed set was lower than the MAALs, while the selfed MAALs had lower rates than the parents. Finally, the rate of silique set for selfed AA+2LGC6 was relatively higher (62.50%) but its rate of seed set was 0.

Discussion

Nuclear–cytoplasmic interactions are important for the evolution of allopolyploids and hybrids (Wendel 2000; Levin 2003).

Table 3. Pollen quantity and viability, and setting rates for siliques and seeds of MAALs and parents.

Material	No. of pollen grains per anther	% Pollen viability	% Silique set	No. of seeds/ (silique × placenta)
B. pekinensis	28,583	86.25	89.12	0.78
B. capitata	41,083	89.12	94.82	0.86
AA+LG2	18,916	79.26	78.28	0.45
AA+2LG2	9167	70.12	67.10	0.25
AA+LG3	11,500	70.78	62.22	0.32
AA+2LG3	9465	68.73	60.36	0.16
AA+LG6	19,666	85.46	18.90	0.14
AA+2LG6	14,833	10.05	62.50	0.00
AA+LG7	25,833	67.55	80.89	0.46
AA+2LG7	12,906	62.78	65.26	0.28
AA+LG8	10,916	76.11	90.61	0.62
AA+2LG8	9333	74.16	32.40	0.10

Cheng *et al.* (2012) showed that pair-crosses of three cultivated diploids have had cytoplasmic and genomic effects on chromosomal recombination and stability in *Brassica* hybrids and allopolyploids. That research group has suggested that the difference in pollen viability between the reciprocal AA.CC (83.89%) and CC.AA (33.33%) might possibly be attributed to cytoplasmic effects. Here, we backcrossed the AAC sesquidiploids (derived from a cross between *B. rapa* ssp. *pekinensis* (AAAA) as female and *B. oleracea* var. *capitata*; CC genome) to *B. rapa* ssp. *pekinensis* (AA) to generate MAALs. As a result, the alien addition lines that carried various C chromosomes showed changes (both up and down) in their vigour when compared with the parent *B. rapa* ssp. *pekinensis*. Heneen *et al.* (2012) reported that, regardless of which chromosome had been added in *B. rapa* var. *trilocularis*–*B. oleracea* var. *alboglabra* MAALs, plants generally were shorter and had diminished vigour and fertility when compared with euploid AA plants. The cause of all those declines might have been cytoplasmic, with the nuclear–cytoplasmic incompatibilities in their MAALs (cytoplasmic of *B. oleracea* var. *alboglabra*, a set of chromosomes from *B. rapa* var. *trilocularis* and one from *B. oleracea* var. *alboglabra*) being stronger than in our MAALs (cytoplasmic of *B. rapa* ssp. *pekinensis*, a set of chromosomes from *B. rapa* ssp. *pekinensis* plus one from *B. oleracea* var. *capitata*).

In our investigation, we found one seedling that was $2n = 21$, in which three of the five LG-specific markers from LGC4 had amplified *B. oleracea* var. *capitata*-specific SSR loci and/or alleles. Their production of the other 39 LG-specific markers was consistent with *B. rapa* ssp. *pekinensis*. Karyotype analysis showed that the alien chromosome was metacentric. Although shorter than chr 1, the alien was longer than chr 2 of *B. rapa* ssp. *pekinensis*. It also closely matched chr 2 of *B. oleracea* var. *capitata* in size and centromere position (figure 2h). Future research is needed to identify this alien chromosome by more LG-specific markers of LGC4. Plants of the AA+LGC4 bolted easily after vernalization and had more floral branches than the parent. By contrast, bolting occurred later in plants of the AA+LGC2 and they had fewer floral branches. Therefore, we might conclude that the alien chromosome of AA+LGC2 is more likely chr 3 of *B. oleracea* var. *capitata*.

We also determined that none of the *B. oleracea* var. *capitata*-specific SSR loci and/or alleles was amplified in the single $2n = 21$ seedling. This might have made it trisomic, i.e., the added chromosome was possibly derived from *B. rapa* ssp. *pekinensis*. Previously, Liu (2008) suggested that this additional chromosome was chr 6 of *B. rapa* ssp. *pekinensis* based on karyotype analysis. The pairing of homologous chromosomes was perhaps interrupted by the chromosome from *B. oleracea* var. *capitata*, which led to the trisomic status ($2n = 21$). In fact, trisomic plants of *B. rapa* have been reported among the progeny of selfed *B. rapa*–*B. alboglabra* addition lines (Chen *et al.* 1992; Cheng *et al.* 1994; Heneen and Jørgensen 2001).

We found four LGC6-specific markers and one LGC2-specific marker in AA+LGC6 and AA+2LGC6. Three of the four LGC8-specific markers amplified *B. oleracea* var. *capitata*-specific SSR loci and/or alleles in AA+LGC8 and AA+2LGC8. However, the other LGC8-specific marker (BoE116) amplified the same product as *B. rapa* ssp. *pekinensis*. Geleta *et al.* (2012) also reported two SSR markers from LGC4 and two SSR markers from LGC9 that amplified *B. oleracea* var. *alboglabra*-specific SSR loci and/or alleles. There, the MAAL had the additional chr 1 from *B. oleracea* var. *alboglabra*. This variation in results might be explained by differences in MAALs as well as the materials used for mapping. It is also possible that chromosome structural variations might occur in some cells, e.g. through the deletion and/or chromosome translocations that are interspecific and/or intraspecific of *B. oleracea* var. *capitata*. In AA+LGC2, the amplified products of BoE020 were of two types: those that were of same length as the parents and those that were longer than the parent. The second type might have arisen due to nonreciprocal translocation that occurred at the BoE020 loci in the chromosome from the AAC hybrid. In fact, Gaeta *et al.* (2007) described homologous nonreciprocal transpositions and deletions in resynthesized *B. napus* that were correlated with amplified fragment length

polymorphisms (AFLP) and qualitative changes in the expression of specific homologous genes.

We established correlations between chromosomes and LG designations of *B. oleracea* var. *capitata*. These were in accord with previous work by Howell *et al.* (2002), which integrated the cytogenetic and genetic linkage maps of *B. oleracea* var. *alboglabra*. However, our results differed in that, although Howell *et al.* (2002) showed a correspondence between LGC2 and a slightly shorter chromosome with the 25S rDNA gene small signal, our correspondence was between LGC2 and a slightly longer chromosome. The discrepancy might be due to karyotype analyses that used different materials or mitotic stages, especially since both studies noted that the chromosomes had similar lengths and arm ratios. Geleta *et al.* (2012) also used 25 markers to identify, in only one MAAL, the added alien LG in *B. rapa* var. *trilocularis – B. oleracea* var. *alboglabra* that amplified *B. oleracea* var. *alboglabra*-specific SSR loci and/or alleles. Each LG had one to five markers. Inconsistent with our correspondence, Heneen *et al.* (2012) established that LGC2 matched C7 (i.e., the smallest chromosome reported by Cheng *et al.* (1995) in their study), and that the centromere had a more median position in the idiogram of LGC2 than in its corresponding C7.

During the creation of a MAAL, chromosomal variations, interspecific and/or intraspecific can occur in some loci. Therefore, for improved accuracy when identifying an alien group, the number of specific markers that are assigned to a particular LG should not be too few. Future research will include identifying the short segment of *B. oleracea* var. *capitata* in the *B. rapa* ssp. *pekinensis* genetic background. By doing so we will also require the development of more LG-specific markers. Using information from the genome sequencing of *B. rapa* ssp. *pekinensis* (Wang *et al.* 2011) and *B. oleracea* var. *capitata* (Liu *et al.* 2014), we have already compared 1456 LG-specific Indel markers with *B. rapa* ssp. *pekinensis*. With an evenly distributed *B. oleracea* var. *capitata* LG, we could assign every LG of *B. oleracea* var. *capitata* using more than 100-specific Indel markers.

In addition, we will further backcross and/or selfcross these MAALs through several generations to obtain CSILs. These CSILs can then be used to create new germplasm and varieties by introducing beneficial genes from related species. The most typical example already adopted is carrying resistance and high-yield genes (1RS) from rye. It has been introduced into wheat and widely applied to the breeding of the crop in numerous countries (Ren *et al.* 2009). These CSILs are also excellent tools for detection and fine-mapping of quantitative trait loci (QTL) for target traits (Ebitani *et al.* 2005; Wang *et al.* 2012). In particular, they can be used to detect QTLs with smaller effects that are easily masked by QTLs that have larger effects in primary populations such as F_2 populations and recombinant inbred lines (Keurentjes *et al.* 2007; Takai *et al.* 2007; Guo *et al.* 2013). To date, many QTLs for biological and economic traits have been detected via CSILs. Some have already been well characterized in studies of β-glucan content in wheat, as well as panicle size and seed dormancy in rice (Mei *et al.* 2006; Cseh *et al.* 2011; Salem *et al.* 2012).

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (31101552, 30871713), the Research Fund for the Doctoral Programme of Higher Education in China (20101302120006) and the Natural Science Foundation of Hebei Province (C2014204093). The authors are grateful to Priscilla Licht for critical reading of the manuscript.

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Received 8 November 2014, in revised form 2 March 2015; accepted 9 March 2015
Unedited version published online: 13 March 2015
Final version published online: 11 September 2015